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Enhanced Biotransformation of Furfural and Hydroxymethylfurfural by Newly Developed Ethanologenic Yeast Strains

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Abstract

Furfural and hydroxymethylfurfural (HMF) are representative inhibitors among many inhibitive compounds derived from biomass degradation and saccharification for bioethanol fermentation. Most yeasts, including industrial strains, are susceptible to these inhibitory compounds, especially when multiple inhibitors are present. Additional detoxification steps add cost and complexity to the process and generate additional waste products. To promote efficient bioethanol production, we studied the mechanisms of stress tolerance, particularly to fermentation inhibitors such as furfural and HMF. We recently reported a metabolite of 2,5-bis-hydroxymethylfuran as a conversion product of HMF and characterized a dose-dependent response of ethanologenic yeasts to inhibitors. In this study, we present newly adapted strains that demonstrated higher levels of tolerance to furfural and HMF. *Saccharomyces cerevisiae* 307-12H60 and 307-12H120 and *Pichia stipitis* 307 10H60 showed enhanced biotransformation ability to reduce HMF to 2,5-bis-hydroxymethylfuran at 30 and 60 m*M*, and *S. cerevisiae* 307-12-F40 converted furfural into furfuryl alcohol at significantly higher rates compared to the parental strains. Strains of *S. cerevisiae* converted 100% of HMF at 60 m*M* and *S. cerevisiae* 307-12-F40 converted 100% of furfural into furfuryl alcohol at 30 m*M*. The results of this study suggest a possible *in situ* detoxification of the inhibitors by using more inhibitor-tolerant yeast strains for bioethanol fermentation. The development of such tolerant strains provided a basis and useful materials for further studies on the mechanisms of stress tolerance.

Index Entries: Hydroxymethylfurfural; 2,5-bis-hydroxymethylfuran; biotransformation; furfural; furfuryl alcohol.

Introduction

With the ever-increasing need for alternative energy sources, the significance of agriculture as an energy producer has been recognized.

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Renewable biomass including lignocellulosic materials and agricultural residues has become an attractive potential low-cost feedstock for bioethanol production *(1,2)*. Biomass pretreatment using acid hydrolysis generates inhibitory compounds, which interfere with the subsequent fermentation. Among more than 100 compounds detected, furfural and hydroxymethylfurfural (HMF) are the most potent and representative inhibitors *(3–5)*. These compounds damage cell growth, reduce enzymatic and biologic activities, break down DNA, and inhibit protein and RNA synthesis *(6–8)*. The inhibitory effects of furfural and HMF on yeasts were documented and appeared to be dose dependent *(9)*.

Fermentation-based bioprocesses rely extensively on improved performance of microorganisms. Genetically modified yeast strains have shown enhanced capabilities for ethanol fermentation through improved utilization in a broad range of applications *(10–13)*. However, few yeast strains genetically improved for inhibitor tolerance are available owing to a lack of understanding of mechanisms involved in the stress tolerance of microorganisms for bioethanol fermentation. Strains and mutations tolerant to biomass fermentation inhibitors are needed for studies of tolerance mechanisms and future development of novel strains. In our previous study, we observed an adaptive response of yeasts to furfural and HMF at tolerable dosages. We developed several strains of yeasts using a directed adaptation strategy. An adapted strain of *Pichia stipitis* was previously reported to have improved performance in ethanol production using hemicellulose hydrolysate *(14)*.

A significant amount of knowledge has been accumulated about yeast stress tolerance for alcohol fermentation, especially for osmotic stress including recent genome expression analysis in environmental stress response *(15–18)*. By contrast, relatively few data are available regarding the inhibitory stress of furfural and HMF involved in bioethanol fermentation. To promote efficient bioethanol production, we are studying stress tolerance mechanisms to improve the performance of ethanologenic yeasts. In a previous study, we demonstrated dose-dependent inhibition of furfural and HMF to ethanologenic yeasts, and varied adaptation abilities of the yeasts to the inhibitors under defined cultural conditions. We reported the first identification of a metabolite, 2,5-bis-hydroxymethylfuran (furan-2, 5-dimerhanol, FDN) as a biotransformed conversion product of HMF by yeasts *(9)*. In the present study, we improved biotransformation by newly developed strains adapted to tolerate the challenges of furfural and HMF in batch cultures compared with the parental strains. The results suggest a possible *in situ* detoxification of the inhibitors for bioethanol fermentation using improved yeast strains. The development and study of such strains provided necessary materials for further studies of the mechanisms of the stress tolerance at molecular and genomic levels.

Materials and Methods

Yeast Strain, Medium, and Culture Conditions

P. stipitis NRRL Y-7124 and *Saccharomyces cerevisiae* NRRL Y-12632 were obtained from the Agricultural Research Service Culture Collection (Peoria, IL). Recently developed more tolerant strains of *P. stipitis* (307-10H60), and *S. cerevisiae* (307-12H60, 307-12H120, and 307-12F40) using directed adaptation strategy were used. Basic medium components were supplied by Difco (Detroit, MI), and amino acids and all chemicals used were provided by Sigma-Aldrich (St. Louis, MO).

Cultures were routinely maintained and cultured on a synthetic complete medium consisting of 6.7 g of yeast nitrogen base without amino acids and 20 g of dextrose supplemented with 16 amino acids. The amino acid components were added into the medium aseptically at final concentrations of 20 mg/L of adenine sulfate, 20 mg/L of uracil, 20 mg/L of L-tryptophan, 20 mg/L of L-histidine hydrochloride, 20 mg/L of L-arginine hydrochloride, 20 mg/L of L-methionine, 30 mg/L of L-tyrosine, 30 mg/L of L-Leucine, 30 mg/L of L-isoleucine, 30 mg/L of L-lysine hydrochloride, 50 mg/L of L-phenylalanine, 100 mg/L of L-glutamic acid, 100 mg/L of L-aspartic acid, 150 mg/L of L-valine, 200 mg/L of L-threonine, and 400 mg/L of L-serine.

Inoculate culture was prepared using freshly grown cells harvested at logarithmic growth phase and incubated with agitation of 250 rpm for 16 h at 30°C. Fermentation cultures were inoculated with 1% of the inoculate culture each separately and grown in 300 mL of synthetic medium in a fleaker system with agitation at 30°C. For inhibitor-treated cultures, the media were amended with either 30 or 60 m*M* HMF, or 30 m*M* furfural. Cultures without inhibitors served as a control. Cultures were monitored for growth at OD_{600} and samples were collected periodically. At least two replicated experiments were carried out for each treatment and sample collection.

Adaptation of Yeast Strains to Furfural and HMF

A directed adaptation method was developed and applied. Strains were first grown in a synthetic medium containing low concentrations of either furfural or HMF tolerable to parental strains of *P. stipitis* NRRL Y-7124 or *S. cerevisiae* NRRL Y-12632. Surviving cells grown in a liquid broth containing inhibitor were transferred into a fresh medium broth amended with the inhibitor once logarithmic growth phase was reached. Cultures were monitored and subsequently transferred iteratively in the same manner. As the adapted cultures became stable, inoculum level was gradually reduced. Once the adapted culture was established, it was then introduced into a medium with a higher concentration of the inhibitor.

This iterative process was upgraded to higher inhibitor concentrations until a desirable tolerance level was reached. For each of the adapted strains, selection and subculture transferring were carried out at least 100 times to obtain a relative uniform and stable population. Stable cultures were maintained using no more than 1% inoculum (v/v) in the respective inhibitory medium and stored in the synthetic broth with glycerol at −80°C.

High-Performance Liquid Chromatography Analysis of Fermentation Kinetics

Samples were analyzed for glucose consumption, ethanol production, furfural, and HMF conversion using a Waters high-performance liquid chromatography (HPLC) equipped with an Aminex Fast Acid column or an Aminex HPX-87H column (Bio-Rad, Hercules, CA) and a refractive index detector. The column was maintained at 65°C and samples were eluted with 5 m*M* H_2SO_4 at 0.6 mL/min. HPCL analysis was standardized using solutions of pure compounds including furfuryl alcohol obtained from Sigma-Aldrich or via the isolation method as previously described for FDM *(9)*, which is not commercially available.

Results

Under the challenge of 30 m*M* HMF in a synthetic medium, both adapted strains 307-12H60 and 307-12H120 of *S. cerevisiae* grew quickly into a stationary phase in 16 h (Fig. 1). By contrast, control strain Y-12632 showed a 34-h lag time of cell growth and reached the stationary phase 48 h after incubation. The metabolic profiles of the control strain showed significant delays of glucose consumption, ethanol production, and HMF conversion in the presence of HMF compared with those without HMF as measured by HPLC analysis (Fig. 2A,D). In the absence of HMF, ethanol production increased with the consumption of glucose for the control strain; however, in the presence of 30 m*M* HMF, glucose consumption was delayed to about 34 h, when it proceeded quickly such that glucose had disappeared by 48 h. HMF levels were also observed to decrease as cell biomass (OD_{600}) increased, and the glucose decreased until HMF was finally converted into bis-hmf 48 h after incubation. Concentrations of bis-hmf, a metabolite of HMF, increased with decreasing concentrations of HMF over time. At 48 h after incubation, HMF was not detectable. Both adapted strains showed rapid glucose consumption and reached the highest potential of ethanol yield in 16 h regardless of whether HMF was present or not (Fig. 2B,C,E,F). HMF had disappeared in the 307-12H60 culture by 16 h, but a small amount of HMF remained in the 307-12H120 culture until 30 h after incubation. Concentrations of bis-hmf increased with decreasing concentrations of HMF over time.

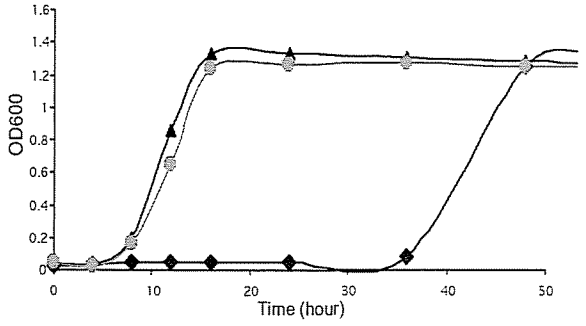

Fig. 1. Comparative cell growth of *S. cerevisiae* NRRL Y-12632 (control, ◆), and newly developed strains 307-12H60(●) and 307-12H120(▲) as measured at OD_{600} on defined synthetic medium containing 30 m*M* HMF. The extended lag phase indicates the inhibitory effect of HMF on the control.

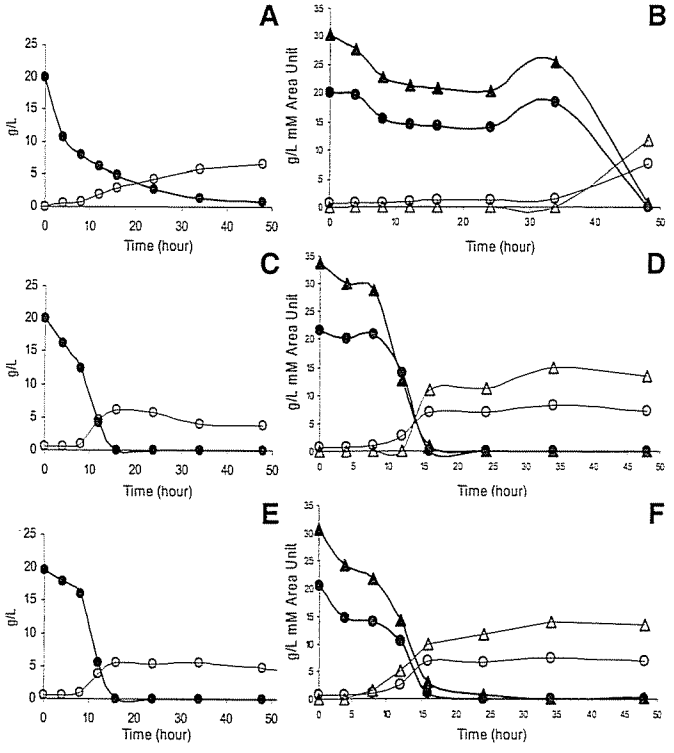

Fig. 2. Glucose consumption(●) and ethanol production(○)in synthetic medium in absence (**A–C**) or presence of 30 m*M* HMF (**D–F**) for strains NRRL Y-12632 (**A, D**), 307-12H60 (**B, E**), and 307-12H120 (**C, F**) showing effects of HMF on different strains. In the presence of HMF, FDM, a metabolite transformed from HMF, was produced at varied rates and concentrations for different strains during the fermentation. Concentrations of glucose and ethanol were estimated by HPLC in grams/liter, HMF in millimolar, and FDM in area units (AU) (1 AU = $1.5^{*}10^{4}$ mAU-min), and the csell growth was monitored at OD_{600}.

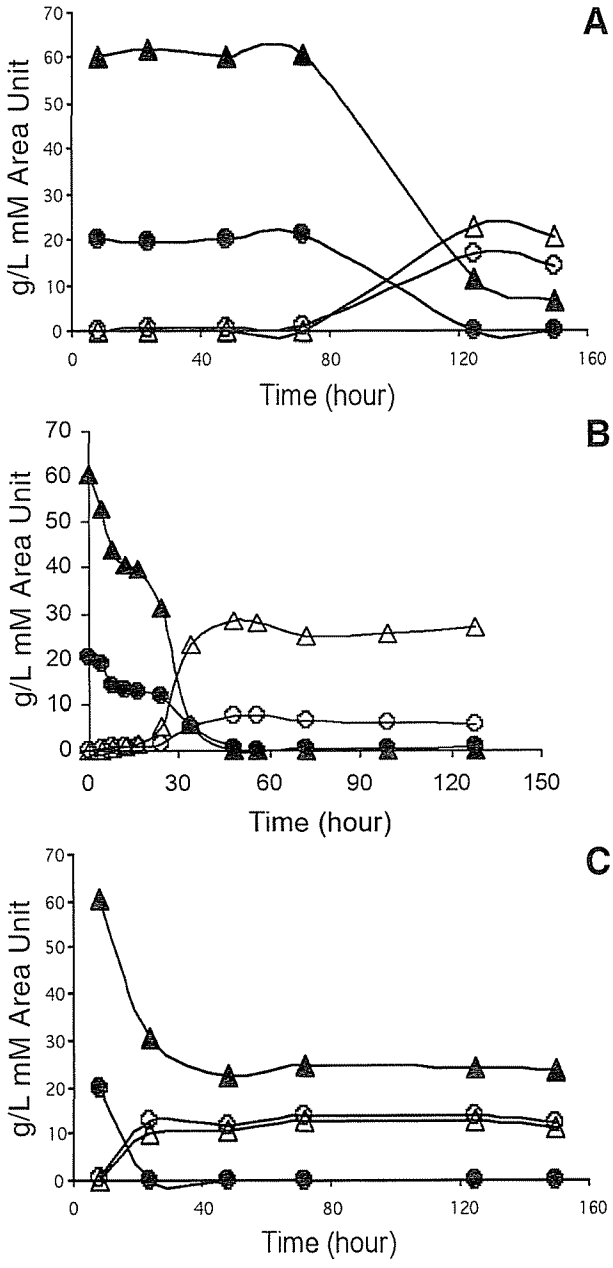

Fig. 3. Glucose consumption(●), ethanol production(○), HMF(▲), and FDM(Δ) recovery for *S. cerevisiae* NRRL Y-12632 **(A)**, 307-12H60 **(B)**, and *P. stipitis* 307-10H60 **(C)** in defined synthetic medium containing 60 mM HMF. Concentrations of glucose and ethanol were estimated by HPLC in grams/liter, HMF in millimolar, and bis-hmf in AU (1 AU = 1.5×10^4 mAU-min).

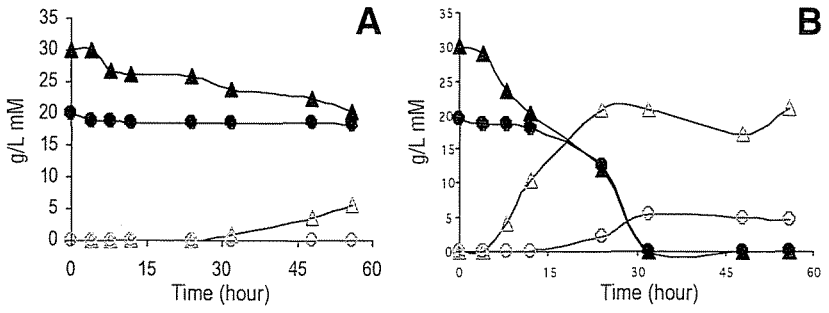

Fig. 4. Glucose consumption(●), ethanol production(○), furfural(▲), and furfuryl alcohol recovery(Δ) for *S. cerevisiae* NRRL Y-12632 **(A)** and 307-12F40 **(B)** in defined synthetic medium containing 30 m*M* furfural showing enhanced transformation of furfural and ethanol production compared with control. Concentrations of glucose and ethanol were estimated by HPLC in grams/liter, and furfural and furfuryl alcohol in millimolar.

When 60 m*M* HMF was applied, control strain *S. cerevisiae* responded with an extended lag phase in cell growth (data not shown) and corresponding delays of glucose consumption, ethanol production, and HMF conversion for at least 72 h after incubation (Fig. 3A). In addition, HMF was still detectable at the end of the fermentation even 6 d after incubation. Strain 307-12H60 reached the maximum potential for glucose consumption, ethanol production, and HMF conversion 48 h after incubation. HMF was not detectable after 48 h for 307-12H60 (Fig. 3B). *P. stipitis* 307-10H60 responded with a rapid glucose consumption and ethanol production. It also showed a bis-hmf recovery with decreased concentrations of HMF (Fig. 3C). However, a significant amount of HMF remained detectable throughout the course of the fermentation. The parental strain NRRL Y-7124 was not able to recover in cell growth from treatment 60 m*M* HMF with (data not shown).

In the presence of 30 m*M* furfural, strain Y-12632 did not show significant glucose consumption and no ethanol production was detectable at 56 h after incubation (Fig. 4A). However, furfural concentration showed a slight decrease, and a small amount of furfuryl alcohol was detectable at 56 h. For strain 307-12F40, glucose consumption and furfural conversion were completed, and the maximum ethanol production potential and furfural alcohol recovery were reached at 30 h after incubation (Fig. 4B). Cell growth of the adapted 307-12F40 showed less suppression by furfural than did the parental control culture, a result similar to that found with the HMF-treated control and adapted strains (data not shown).

Discussion

Based on observations of a dose-dependent yeast response to furfural and HMF, we developed a directed adaptation method and generated several strains more tolerant to furfural and HMF. We evaluated these adapted ethanologenic yeast strains and demonstrated their significantly higher levels of tolerance to HMF and furfural compared with the parental strains under controlled conditions. These adapted, more tolerant strains showed no significant delay in cell growth and glucose consumption. They produced normal yield of ethanol in the presence of furfural or HMF. More important, these adapted strains were more tolerant to the inhibitors and showed enhanced biotransformation ability to convert furfural into furfuryl alcohol and HMF into FDM compared with that of the parental strains.

In our previous report on the dose-dependent response of yeasts to the inhibitors, we hypothesized that a cell adaptation process could occur during the extended lag phase before the active cell growth and metabolism recovered *(9)*. In the present study, we demonstrated the nearly normal growth of adapted strains in the presence of HMF and furfural, which indicated a qualitative change in cell response to the presence of the inhibitors compared with the parental strains. The adapted strains appeared to be distinct with their original parental strains in both growth response and metabolic profiles, as measured by HPLC. Microorganisms including yeasts live in an ever-changing environment and must constantly adapt to specific environmental changes for survival. As documented in numerous reports, yeast adaptation to a stress condition is common and accomplished via a variety of molecular mechanisms *(19,20)*. Global gene expression analysis supports the existence of yeast adaptation responses to stress conditions *([18]*; unpublished data). An adapted *P. stipitis* was reported to have improved ethanol production from hemicellulose hydrolysate *(14)*. It appeared that adaptation can be an alternative means to improve microbial strains. Our directed adaptation method reported herein favored a selection of mutants or adapted populations that were tolerant to furfural and/or HMF. Further improvements in this method may enhance the process of evolution to accomplish needed stress adaptation for a broad range of applications.

Pulse addition of furfural and HMF has been used to study the effects of the inhibitors to yeasts *(4)*. Using this method, cell growth is initiated without the inhibitor, and the inhibitor is added into the culture when a desired cell population has been established. In such a method, yeasts show a phenomenon of a slight repression followed by a recovery of active cell growth and metabolism. There is a short pause in cell metabolism but no significant lag phase. By contrast, a significant lag phase was observed when the inhibitors were present initially in batch cultures *(9)*. As indicated in the present study, when there was no cell growth, no HMF conver-

sion occurred. This suggested that the HMF conversion into FDM is a biologic activity carried out by the yeasts. It appeared that an established population of yeast in a pulse-addition method may help to reduce the degree of the inhibitory stress by conversion of HMF into a less toxic form of FDM. Actually, this method has been suggested as an alternative to overcome the inhibitors for biomass fermentation *(4)*. Strains that performed well under such conditions did not tolerate the inhibitors in batch cultures. The adapted strains used in our study grew immediately as initiated in a batch culture in the presence of the inhibitors. They demonstrated an active metabolism to consume glucose, produce ethanol, and transform HMF into FDM. This response of these strains in a diluted inoculate into an HMF- or furfural-containing medium is different from that of a dense, grown population exposed to a pulseaddition of the inhibitors. Mechanisms involved in such different responses may not be identical. Our preliminary results of genomic expression analysis support yeast adaptation response to the inhibitors.

The adapted strains were able to convert 100% of HMF into FDM at either 30 or 60 m*M* and produce normal yields of ethanol. Similar to the HMF-tolerant strains, 307-12F40 converted 100% of furfural into furfuryl alcohol and produced ethanol efficiently. This suggested a potential for *in situ* detoxification of the inhibitors by using HMF- or furfural-tolerant yeasts for ethanol production. Unlike adapted strains of *S. cerevisiae*, strain 307-10-H60 of *P. stipitis* showed only about 60% conversion of HMF at a fermentation condition of 60 m*M* HMF. As shown for the adapted *S. cerevisiae* strain 307-12H60, 60 m*M* HMF slowed down the conversion rate of HMF compared with that of 30 m*M*. Apparently, the dose of inhibitor is a key factor of concern, and further study is needed to describe the inhibitor dosage limit for a given strain to be potentially used for *in situ* detoxification. *P. stipitis* is a natural xylose utilization yeast and would be a valuable resource for bioethanol fermentation. On the other hand, *S. cerevisiae* appeared to be more tolerant of a wide range of inhibitors (unpublished data). Both yeasts have potential to be improved for further development of HMF- and furfural-tolerant strains.

Our adapted strains tolerant to furfural and HMF showed significant enhancement of biotransformation to reduce these inhibitors, which suggested a potential for *in situ* detoxification of inhibitors using tolerant strains for more efficient bioethanol production. These adapted, more tolerant strains pose relatively high levels of tolerance to single inhibitors. However, they have not been tested against inhibitor complexes such as those in a biomass hydrolysate and need further improvement for use in biomass fermentation applications. Single inhibitor-tolerant strains are necessary for studies dissecting the mechanisms of stress tolerance to the multiple inhibitor complexes. These adapted strains will be a valuable resource in our upcoming studies of molecular mechanisms of stress tolerance using functional genomics.

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